Evaluation the Levels of Yap1 Protein and NF-Kb in Serum of Women with Breast Cancer

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KEYWORDS
Breast cancer; Inflammatory, Oncoprotein; NF-κB; Yap1

ABSTRACT
Breast cancer (BC) is the type of cancer that is most frequently diagnosed and is the primary cause of cancer-related deaths in women globally. BC is intimately linked to both apoptosis and inflammation. This study aimed to evaluate NF-kappaB (NF-κB) and Yes-associated protein (Yap1) biomarkers in the serum of women patients with (BC).

This case-control research recruited a total of 90 women, consisting of 60 women diagnosed with breast cancer and 30 healthy controls of the same age. This research detected a statistically significant rise ($P<0.05$) in the levels of NF-κB and Yap1 in the serum of the BC group compared to the control group. from the results of Receiver operating characteristic (ROC) analysis, the present results indicate an optimal NF-κB cut-off value of more than 3.06 ng/ml resulted in an AUC value of 0.934. And is considered an excellent diagnostic marker, also. An optimal Yap1cutt-off value of more than 13.72 ng/ml resulted in an AUC value of 0.997 Yap1, while There was no significant difference $P>0.05$ in NF-κB and Yap1 levels among BC women according to clinical-pathological, molecular type and phenotype clinical and pathological characteristics.

We conclude that These results indicate the roles of NFκB and Yap1 as biomarkers for breast cancer diagnosis.

1. Introduction

In 2020, there were 2.2 million new instances of breast cancer recorded globally, with emerging nations accounting for more than 50% of the disease's fatality rate [1]. defined by varying outcomes based on genetic profile and histological features [2]. Breast cancer can be divided into at least three subtypes based on the expression profile of its gene: luminal tumors, which are positive for progesterone receptors (PR+) and/or estrogen receptors (ER+); HER2-enriched tumors, which overexpress the ERBB2 oncogene; and triple-negative tumors (TNBC), which do not have HER2 amplification or hormone receptors. Regarding incidence, response to therapy, chance of disease progression, and metastasis sites, each subtype varies [3]. Females with a first-degree relative who has been diagnosed with BC are more likely to experience the condition [4]. The main risk factors for BC are hormone-containing birth control pills, obesity, low nutrition, physical inactivity, hormone replacement treatment, alcohol use, and smoking. Hereditary and genetic factors have a significant role in the development of breast cancer. Research has shown that 5–10% of instances are caused by mutations in the BRCA1 and BRCA2 genes, and that encouraging breastfeeding and physical activity may lower risk [5].NF-κB1 is a transcription factor that regulates several biological processes. Research has shown that NF-κB1 plays a vital role in regulating immune responses. [6] In humans, the NF-κB superfamily comprises five transcription factors: NF-κB1 (p50), NF-κB2 (p52), RelA (p65) RelB, and REL (c-Rel) [7]. Research on the signalling pathways involved in NF-κB activation is extensive. The canonical and noncanonical NF-κB activation signalling routes are the two different kinds [8]. Each depending on unique chemical mechanisms to activate the many sets of target genes involved in immune response, differentiation, and cell proliferation in addition to NF-κB.[9]. The majority of malignancies exhibit higher NF-κB activation, which is frequently brought on by increased NF-κB pathway stimulation, such as elevated TNFα and IL-1 within the milieu of the tumor [10]. Conversely, NF-κB has a definite function that suppresses tumour growth that has been shown in mice and tumour cell line models, but has not yet been fully shown in human cancers [11]. The regulation of cell survival is one of the NF-κB's key roles. NF-κB activation typically results in decreased cell apoptosis in tumour cells [12]. The potential of NFκB as a therapeutic target and a diagnostic biomarker has made its participation in breast cancer an important area of study emphasis. The complex link between NF-κB and breast cancer involves several upstream signals, including as proinflammatory cytokines, DNA damage, and growth factors, which are frequently present in the tumor microenvironment[13].As a downstream effector of the Hippo pathway, yes-associated protein (Yap1) regulates proliferation, which in turn governs organ growth, normal tissue homeostasis, differentiation, and apoptosis of normal stem cells [14].YAP is often expressed in the cytoplasm and nucleus of cells. But YAP only carries out its biological activities when it is inside the nucleus. It does this primarily by binding to transcription factors, such as transcriptional enhancer activator domain (TEAD), which controls drug resistance, oncogenic transformation, cell proliferation, and the epithelial-to-mesenchymal transition (EMT)[15]. The human Hippo pathway involves the successive phosphorylation of Lats1/2 kinases and YAP by activation of Mst1/2 kinases. By attaching to 14-3-3 proteins, activated Lats kinases phosphorylate YAP mostly at the Ser127 location (S61, S109, S164, S381, T63, S138, S289, S351, and S384), which helps to keep YAP in the cytoplasm. Because of this, the pYAP-S127 level is frequently used to gauge the activity of the Hippo pathway [16]. YAP is involved in the regulation of neoplasm initiation, growth, metastasis, and medication resistance in tumors. Crucially, YAP is overexpressed and undergoes nuclear translocation in a variety of tumour types [17].It has been shown that Yap1 stimulates the growth, spread, and metastasis of numerous solid tumors[18]. A study found that Yap1 acted as a tumor suppressor in breast cancer [19].

2. Material and Methods

Study Design and Blood Sample Collection

this study was a case-control study included 60 women who were diagnosed with BC by a specialist doctor based on clinical syptoms and the results of the Magnetic Resonance Imaging in addition, 30 healthy control groups who had no history or clinical evidence of BC. The patients were recruited from the Wasit Specialized Center for Oncology, while the control group was from Al Karama Teaching Hospital. Wasit City, Iraq. All information about recovery patients and control group was noted in questionnaire forma during direct meeting with patients and control individuals, the questionnaire forma, which contain name, age, obesity, and Family History or not. The Clinical and pathological characteristic of BC including (Stage, Grade, Position and treatment in addition to molecular subtype (ER,PR,HER2) and phenotype) were diagnosed by the doctors by using many methods. (5ml) of blood was obtained from total individuals, then placed in a gel tube. which left to clotted at room temperature (20- 25 ºC) for 30 minutes. The serum was separated by a centrifuge at a speed of (5000 rpm) for 10 minutes and kept at -20 ˚C to determine the levels of NF-κB andYap1.

ELISA analysis:

ELISA techniques have been used to determine the quantitative of NF-κB and Yap1 levels in serum samples of patients women with BC and healthy control according to the manufacturer's instructions. China's Bioassay Technology Laboratory offered human ELISA kits.

Statistical analysis

Data analysis was conducted using SPSS 26 and Excel 2010. Variables are characterized by mean, standard deviation, and standard error. Based on the results of the independent sample t-test, the difference in mean values between the two groups was measured. Using the one-way analysis of variance (ANOVA) test, the differences in average values between the several groups were evaluated. An examination of the relationship between two category variables was carried out with the help of the chi-square ($\chi2$) test. The risk was evaluated by using the odds ratio in conjunction with a confidence interval of 95%. The examination of the receiver operator characteristic (ROC) curve, together with its associated area under the curve (AUC), accuracy level, sensitivity, specificity, and degree of significance (P), was carried out in order to ascertain the cutoff value that was capable of predicting a good outcome.Both the odds ratio and the confidence interval with a 95% level were calculated in order to measure risk. In order to determine the importance of the findings, a P-value threshold of less than 0.05 was used, with values of 0.01 or below being regarded as more significant.

3. Result and Discussion

Comparison of BC patients' NF-κB and Yap1 levels with healthy controls.

The levels of NF-κB and Yap1 were examined between BC patients and healthy controls. The findings are shown in Figure (1). Patients diagnosed with breast cancer had substantially increase levels of NF-κB compared to those without the disease ($P< 0.001$).The levels of BC patients were measured to be 3.65±0.80 ng/ml ,whereas the levels of healthy controls were 2.51±0.39 ng/ml. The average levels of

Yap1 in patients with breast cancer (BC) and healthy controls were 18.27±1.33 ng/ml and 8.55±3.02 ng/ml respectively. There was a substantial increase in the mean level was highly significant higher than in patients with BC in comparison with healthy control (P< 0.001).

Level of NF-κB (ng/ml) in serum **Level of Yap1 (ng/ml) in serum**

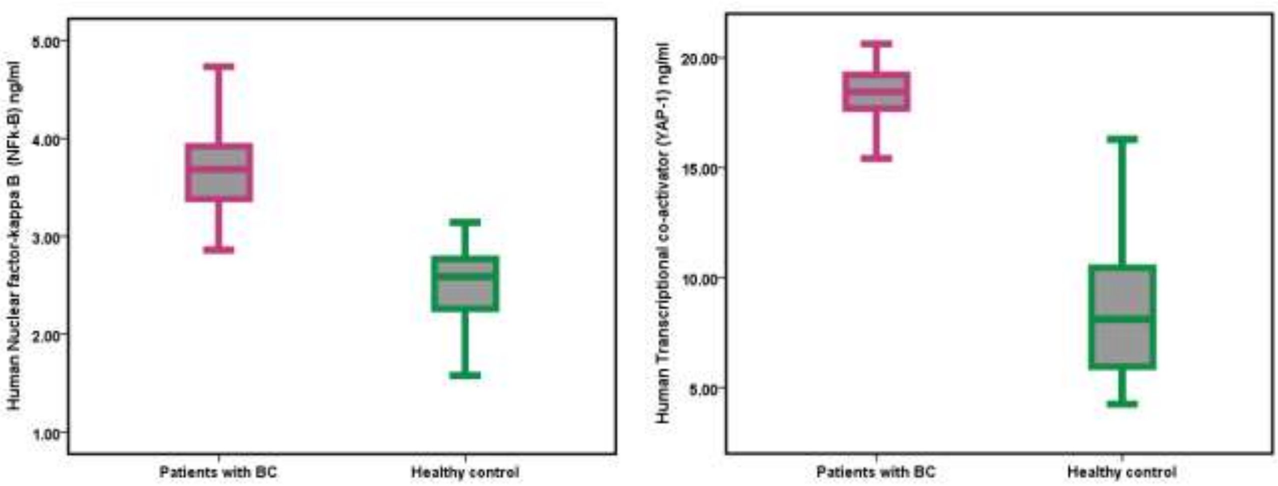


Figure (1): Quantification of NF-κB and Yap1 in serum

Receiver operating characteristic (ROC) analysis for Evaluation of NF-κB and Yap1 levels.
Receiver Operating Characteristic (ROC) analysis was conducted to determine the diagnostic precisi on of using levels of NF-κB and Yap1 in differentiating patients with BC from healthy control participants. The results of this study are shown in Tables 1 and 2, as well as Figure 2 The current findings suggest that NF-κB is deemed an excellent diagnostic marker. While Yap1 is regarded as a superior diagnostic indicator.

Table (1): Sensitivity and specificity of NF-κB (> 3.06-fold) in BC disease

NF-κB levels	**BC patients** ***n* = 60**	**Healthy control** ***n* = 30**
> 3.06	55	1
< 3.06	5	29
Sensitivity %	91.7 %	
Specificity %	96.7 %	
PPV %	98.2 %	

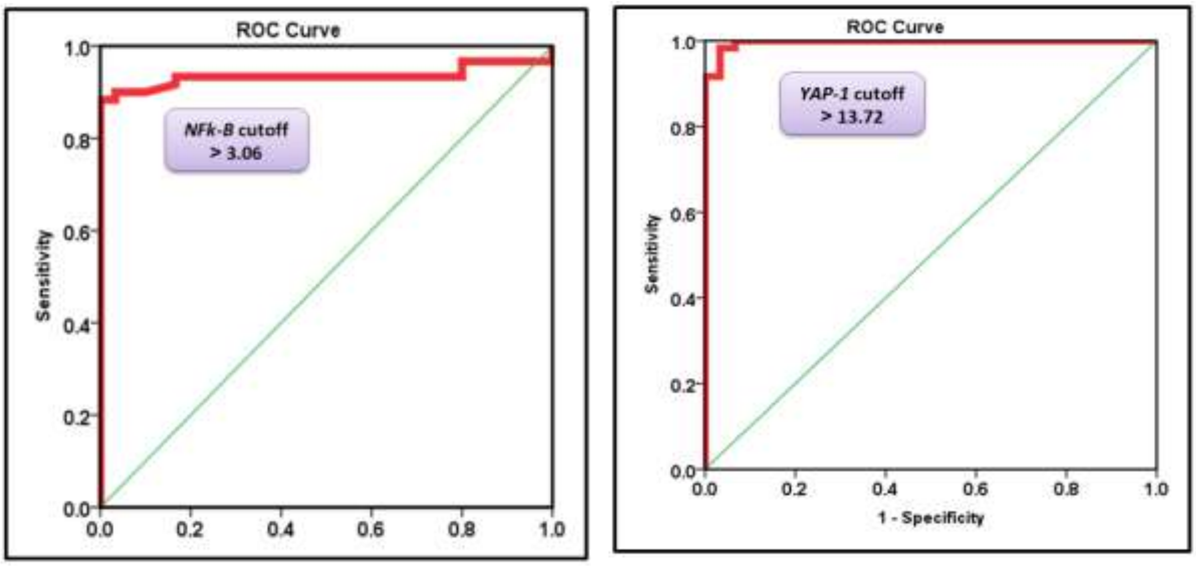
NPV %	85.3%
AUC (95% CI)	0.934 (0.877- 0.992)

CI: Confidence interval, AUC: Area under curve.

Table (2): Sensitivity and specificity of YAP-1 (> 13.72-fold) in BC disease

YAP-1 levels	BC patients *n* = 60	Healthy control *n* = 30
> 13.72	60	1
< 13.72	0	29
Sensitivity %	100.0 %	
Specificity %	96.7 %	
PPV %	98.4 %	
NPV %	100.0%	
AUC (95% CI)	0.997 (0.990- 1.000)	

CI: Confidence interval, AUC: Area under curve



(Figure -2): Receiver operator characteristic curve analysis of NF-κB and Yap1 for the calculation of possible diagnostic cutoff value.

Frequency distribution of NF-κB and Yap1 levels according to some demographic characteristics

Demographic characteristics has been carried out and the results were demonstrated in the table (3). The present results show a non-significant difference in NF-κB and Yap1 levels according to all studied demographic characteristics (P> 0.05).

Table (3) Distribution of NF-κB and Yap1 levels according to some demographic characteristics

Demographic characteristic	N	NF-κB Mean ± SD	*P*	YAP-1 Mean ± SD	*P*

Family History	Positive	8	3.67 ± 0.82	0.704 † NS	18.33 ± 1.39	0.409 † NS
	Negative	52	3.55 ± 0.61		17.91 ± 0.83	
Obesity	Normal	14	3.32 ± 0.98	0.071 A NS	18.09 ± 1.62	0.667 A NS
	Overweight	20	3.56 ± 0.83		18.41 ± 1.26	
	Obese	26	3.91 ± 0.58		18.42± 0.74	

n: number of cases; **SD**: standard deviation; †: independent samples t-test; A: anova test; NS: not significant at P > 0.05.

Frequency distribution of NF-Kb and Yap1 levels according to molecular subtype and phenotype.

The comparison of NF-κB and Yap1 levels according to molecular subtype and phenotype characteristics has been carried out and the results were demonstrated in Table (4). However, the present results show a non-significant difference in NF-κB and Yap1levels according to all molecular subtype and phenotype characteristics (P> 0.05).

Table(4): Distribution of NF-κB and Yap1 levels according to molecular subtype and phenotype

Characteristics		N	NF-κB Mean ± SD	*P*	YAP-1 Mean ± SD	*P*
Estrogen receptor (ER)	Positive	43	3.72 ± 0.72	0.307 † NS	18.39 ± 1.316	0.315 † NS
	Negative	17	3.48 ± 0.97		18.00 ± 1.70	
Progesterone receptor (PR)	Positive	37	3.75 ± 0.76	0.212 † NS	18.33 ± 1.13	0.674 † NS
	Negative	23	3.49 ± 0.84		18.18 ± 1.63	
HER2	Positive	20	3.75± 0.82	0.169 † NS	18.61± 1.39	0.165 † NS
	Negative	40	3.45 ± 0.77		18.10 ± 1.44	
M. Type	Luminal A	30	3.82 ± 0.78	0.354 A NS	18.33 ± 1.18	0.127 A NS
	Luminal B	12	3.48 ± 0.52		18.64 ± 1.09	
	Triple negative	11	3.58± 0.75		17.46± 1.88	
	Her2	7	3.31 ± 1.02		18.67 ± 1.01	
H. Type	IDC	48	3.65 ± 0.84	0.908 A NS	18.30 ± 1.41	0.872 A NS
	ILC	7	3.82 ± 0.29		17.86 ± 1.04	
	DCIS	1	3.08		19.17	
	IDC+DCIS	1	3.76		18.01	
	IDC+ ILC	3	3.43 ± 1.16		18.38 ± 1.20	

n: number of cases; **SD**: standard deviation; †: independent samples t-test; A: anova test; NS: not significant at P > 0.05.

Frequency distribution of NF-κB and Yap1 levels according to clinical and pathological characteristics.

The comparison of NF-κB and Yap1 levels according to clinical and pathological characteristics has been carried out and the results were demonstrated in Table (5). The present results show a non-significant difference in NF-κB and Yap1 levels according to all clinical and pathological characteristics (P> 0.05**).**

Table (5): Distribution of NF-κB and Yap1 level according to clinical and pathological characteristics

Clinical and pathological		N	NF-κB Mean ± SD	*P*	*YAP-1* Mean ± SD	*P*
BC Stage	IA	2	3.42 ± 0.48	0.861 A NS	18.98 ± 1.02	0.596 A NS
	IIA	15	3.71 ± 0.68		17.98 ± 1.26	
	IIB	9	3.75 ± 0.27		17.84 ± 1.85	
	IIIA	10	3.42 ± 0.55		18.46 ± 1.33	
	IIIC	4	4.01 ± 0.76		19.02 ± 1.55	
	IV	20	3.63 ± 1.11		18.38 ± 1.15	
BC Grade	Grade 1	4	4.1 ± 0.75	0.379 A NS	18.91 ± 1.18	0.107 A NS
	Grade 2	46	3.58 ± 0.78		18.07 ± 1.32	
	Grade 3	10	3.81 ± 0.63		18.95 ± 1.23	
BC Side	Right	26	3.48 ± 1.02	0.293 A NS	18.25 ± 1.01	0.903 A NS
	Left	33	3.77 ± 0.56		18.31 ± 1.57	
	Bilateral	1	4.24		17.71	

n: number of cases; **SD**: standard deviation; A: anova test; NS: not significant at P > 0.05.

Regression correlations between YAP-1 and NFk-B

The Logistic regression model of the correlation between YAP-1 and NFk-B of BC patients women is shown in figure (3), the results indicated that significant positive correlation (P>0.05) between YAP-1 and NFk-B in serum of BC women (r =0.292 and P = 0.023) this result might be refer to that breast cancer condition enhance production of YAP-1 is related to NFk-B.

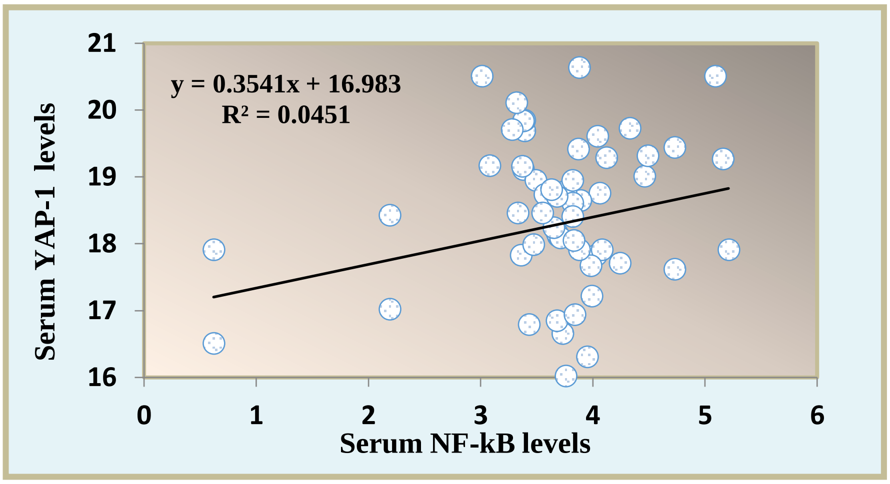


Figure (3) Regression correlations between YAP-1 and NFk-B

Discussion

Cancer can be caused by dysregulation of apoptosis-mediated cell death, which can also impact how well a treatment works [20].In this investigation, we examined NF-κB and Yap1expression, The level of concentrations increased in breast cancer patients compared to healthy controls. However, there are no statistically significant differences in the concentration of the NF-κB level between the clinical and demographic markers of the patients .As a transcription factor, NF-κB plays a crucial part in controlling the processes associated with the development of cancer, including inflammation, cell survival, and proliferation[21].Adiga *et al.* also found that, when compared to healthy individuals, serum NF-κB levels were significantly higher in breast cancer patients, indicating that it may be useful as a biomarker for the disease's diagnosis. In contrast, patients with breast cancer showed very weak relationships with haematological measures such as PLR and NLR[22].Numerous investigations were carried out to ascertain the connection between NF-κB and breast cancer. Swathanthra .found that breast cancer cell overexpressed more NF-κB indicated rising patterns in grades I and II tumors[23].In another study that was carried out on a cohort of Asian female breast cancer patients in India, a connection was discovered between NF-κB expression and clinical stage, high grade, tumor size, high Nottingham prognostic index, HER2,and negative ER status, overexpression [24].Also Cao *et al* found, the luminal subtype exhibited high levels of NF-κB 1 expression. NF-κB 1 expression in the TNBC subtype is greater in the IM subtype compared to other subtypes ($P<0.05$). NF-κB 1 did not correlate with distant metastasis, lymph node stage, or tumour size ($P\geq0.05$[25].Furthermore, in locally progressed breast cancer tissues (17/37), Montagu *et al.* found a 45.9% increase in NF-κB expression. Additionally, it was found that in cases of breast cancer, NF-κB activation predicts treatment resistance [26].One important factor between inflammation and cancer is NF-κB. It has been shown to play a role in both the resistance to endocrine therapy and the carcinogenesis of breast cancer. The control of inflammation, cell line survival, and proliferation are all dependent on NF-κB[27]. Following the formation of a complex with the inhibitor subunits IKB-α, -β, and -ϒ, the nuclear factor kappa B (NF-κB) is discovered in the cytoplasm in an inactive state [28].It is the dissociation of inhibitory subunits (IκB s) that is responsible for the activation and rapid translocation of the NF-κB heterodimer into the nucleus in order to interact with DNA [29].The genes that are targeted by NF-κB and are responsible for inhibiting apoptosis are subsequently expressed when the subunit p65 demonstrates its transcriptional activity. The end effect of this is the construction of a network that regulates the cell cycle and promotes the invasion of cells, inflammation, the growth of tumors, the spread of metastases, and finally the development of resistance to radiation and chemotherapy.Canonical signalling (CS) pathways, which are triggered by LPS, ,ROS,TNF-α, and IL-1, and noncanonical signalling (NCS) pathways, which are triggered by inflammatory stimuli via IKKα, are responsible for regulating NF-κB[30].On the other hand, because NF-κB dysregulation leads to tumour evolution, activated NF-κB is found in a variety of malignancies, including BC[31].Because of its many roles, NF-κB has a very complex role in tumor formation in relation to TME. In solid tumors, NF-κB activation leads to the emergence of inflammatory TME[32].The inflammatory reaction lead to increased cytokine gene expression and cytokine release, which activated the canonical NF-κB signalling pathway and caused altered cells to undergo apoptosis[33].The infiltration of leukocytes causes an increase in breast epithelial cell cancers, which is related to NF-κB signalling [34].For the prognosis and treatment of BC, it can therefore be used as a possible marker and therapeutic target. Despite the cytotoxic impact of medications in eliminating tumors, these treatments have certain drawbacks, such as side effects in many organs, since they target all proliferative and rapidly developing cells, including tumor and normal cells [35]. NF-κB activates BCl-2[36].Bcl-2 family proteins significant regulators of apoptotic cell death via the intrinsic pathway[37].In BC cells, Bcl-2 is activated and overexpressed where it is essential for the development of chemoresistance[38].In various tumor types, Bcl-2 controls the Hippo pathway, which stimulates fibroblast activation, cell migration, and adaptability to stiffer culture conditions[39].YAP functions as a transcriptional co-regulator of the Hippo signalling pathway's activity[40].YAP1 has been described as an oncogene in diverse organs[41].Research has demonstrated that by either directly or indirectly influencing metastasis-associated molecules, YAP/TAZ controls every stage of breast cancer metastasis[42].The potent regulator (YAP) is overexpressed in a large number of malignancies[43].Based on this information, we explain the reason for the high level of YAP concentration in breast patients in the current study,.These outcomes matched those of an Egyptian investigation that discovered YAP1 is significantly expressed and activated in BC and that inhibiting it may offer a fresh approach to treatment[44]. Cha *et al* suggest that nuclear YAP1 expression, particularly in TNBC patients, is a biomarker of a poor prognosis and a possible target for treatment[45]. A study by Ramadan *et al.* found that there was a substantial association between the stage of the cancer and Yap1 methylation, which

was much higher in BC tissue than in normal breast tissue[46].Western blotting, real-time quantitative PCR, and immunohistochemistry assays were previously used to investigate the relationship between YAP1 and BC. It was noted that varying subcellular localization and expression levels [47].Numerous reports came to the overall conclusion that tumor aggressiveness in BC is associated with YAP1 dysregulation [48].Yap activation has a significant impact on the immune system's reduction[49].And the acceleration of the change in cancer cell type [50].A decrease in Yap expression is also linked to diminished stemness or tumour cell senescence[51].It's interesting to note that Yap overexpression or activation is associated with resistance to chemotherapy or radiation in breast cancer[52]. Radiation and chemotherapy depend on raising intracellular ROS, which can trigger apoptosis[53].In BC, YAP is crucial in controlling how cells react to oxidative and metabolic stress. YAP coordinates adaptive processes to improve energy output and food utilization during metabolic stress. It enhances lipid metabolism, glycolysis, and the Warburg effect, which helps cancer cells grow and survive in the absence of nutrients[54].Furthermore, via regulating ROS levels and antioxidant defence systems, YAP supports redox homeostasis. It lessens the harmful effects of oxidative stress on cancer cells by controlling the expression of genes involved in antioxidant pathways [55].
Our findings show that there is a significant positive association between Yap and NF-κB, and that an increase in YAP1 is correlated with an increase in NF-kB. The functional interaction between NF-κB and YAP signalling was discovered by Zhaoa *et al*, who also offered mechanistic insights into the YAP-dependent growth regulation pathway and carcinogenesis[56].The mechanisms that inflammation uses to generate cancer may be the reason of this association. Many forms of cancer have been shown to have a correlation between inflammation and the disease, and inflammation is thought to be the "seventh hallmark of cancer."[57]. It has recently been discovered that YAP, a potential proto-oncogene, interacts with inflammatory signalling during the formation of tumours. One well-known cytokine that controls inflammation during tumour growth is TNFα. An aggressive behaviour and a bad prognosis are linked to high levels of tumour necrosis factor α (TNF α) in various malignant tumours, including breast cancers[58]. Through the activation of IκB kinases (IKKs), c-Jun N-terminal kinase, and mitogen-activated protein kinase signaling through TNF receptor 1 (TNFR1), tumor necrosis factor-alpha (TNFα) induces the movement of transcription factors, including activator protein-1 (AP-1) and nuclear factor kappa B (NF-κB), from the cytoplasm to the nucleus in cancer cells[59]. As an additional point of interest, Gao *et al*. provide evidence that YAP serves as a detector for TNFα. Furthermore, it is shown by them that the activation of both YAP and NF-κB results in a cooperative control of gene transcription in a way that is synergistic.[60].

Conclusion

In this study on the prognostic significance of NF-κB and Yap1 among breast cancer patients, according to our findings, serum NF-κB and Yap1. levels were considerably higher in breast cancer patients than in healthy people, indicating that they may be useful as disease diagnostic biomarkers. In addition, the increase in their levels is evidence of the relationship between them, which is due to the fact that they share different pathways that may play a role in the progression of the breast cancer.

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